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ISOLATION OF CALCIUM CURRENT AND ITS SENSITIVITY TO MONOVALENT CATIONS IN DIALYSED VENTRICULAR CELLS OF GUINEA-PIG

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SUMMARY

1. The ion selectivity of the Ca^{2+} channels in single ventricular cells of guinea-pig was studied using a 'giga-ohm seal' patch electrode for voltage clamp and internal dialysis.

2. To isolate the Ca^{2+} channel current, currents through the Na^+ channel and K^+ channels were minimized by replacing external Na^+ with $Tris^+$ and removing K^+ from both sides of the membrane. With 5 mM-ATP and 5 mM-EGTA in the pipette solution, the Ca^{2+} current was well maintained for more than 30 min in K^+- and/or Na^+-free external solution.

3. Substitution of Cs^+ for intracellular K^+ eliminated the region of negative slope conductance in the steady-state current–voltage curve and shifted the zero-current potential or resting potential from -80 to -31 mV. After Cs^+ substitution, a large inward current still flowed via inwardly rectifying K^+ channels, but was abolished by removing external K^+, which resulted in reduction of the resting membrane slope conductance to 1 % of the control value.

4. A decaying outward current attributable to the inwardly rectifying K^+ channel was observed on depolarization in 5·4 mM-external K^+ solution with Cs^+-rich internal solution after blocking Ca^{2+} current. The induction of that current caused an apparent decrease of Ca^{2+} channel current when the K^+-rich internal solution was switched to the Cs^+-rich one at an external K^+ concentration of 5·4 mM. When inwardly rectifying K^+ current was suppressed by exposure to K^+-free external solution, replacement of intracellular K^+ with Cs^+ caused no significant change in the Ca^{2+} current.

5. With Cs^+-rich solution in the electrode, the decaying outward current was responsible for an apparent depression of the Ca^{2+} current observed when extracellular K^+ was increased. When the K^+ current was abolished by 0·2 mM-extracellular Ba^{2+}, changes in external K^+ concentration did not affect the Ca^{2+} current, excluding the possibility of a direct inhibitory action of K^+ on the Ca^{2+} channel.

6. A time- and voltage-dependent outward current attributed to Cs^+ was observed at potentials above $+30$ mV in Na^+-, K^+-free external solution with Cs^+-rich internal solution. This current persisted in the presence of 20 mM-intracellular TEA Cl and 5 mM-extracellular 4-aminopyridine.

7. Inorganic Ca^{2+} channel blockers, such as Co^{2+} or Cd^{2+}, not only suppressed the inward Ca^{2+} current but also caused some reduction in outward current. Thus the blocker-sensitive peak current reversed at around +75 mV. However, the blocker-sensitive outward current was not inactivated by a conditioning pre-pulse to +15 mV, suggesting that the outward Cs^+ current flows mainly not through Ca^{2+} channels but through some voltage-dependent leakage conductance.

8. The Ca^{2+} channel current was not significantly changed when external Na^+ was replaced with $Tris^+$ in the presence of 3·1 μM-tetrodotoxin. This finding indicates that Na^+ do not contribute significantly to Ca^{2+} channel current.

9. We conclude that neither K^+ nor Na^+ contribute significant charge to the Ca^{2+} current and that, under physiological conditions, the Ca^{2+} channel is believed to be much more selective for Ca^{2+} than previously recognized (Reuter & Scholz, 1977).

INTRODUCTION

Until recently, biophysical studies of Ca^{2+} current in cardiac tissue were hampered by structural complexities of the multicellular preparations and by the difficulty of separating Ca^{2+} current from overlapping membrane current components carried by other ions. Isolated single cells offer a promising system for studying the Ca^{2+} current: they allow good control not only of electrical potential (Isenberg & Klöckner, 1982*b*) but also, if internally perfused, of ionic composition on either side of the membrane (Lee & Tsien, 1982, 1983; Irisawa & Kokubun, 1983).

This paper describes experiments using internal dialysis via the 'giga-ohm seal' patch-clamp electrode (Hamill, Marty, Neher, Sakmann & Sigworth, 1981) to study Ca^{2+} channel current in single ventricular cells from guinea-pig heart. By removing K^+ from both sides of the membrane and replacing external Na^+ with $Tris^+$, we were able to characterize overlapping currents and hence to isolate Ca^{2+} channel current to a considerable degree. Although the persistence of relatively non-specific, time-dependent, outward currents at large positive potentials interfered with precise measurements, estimates of the reversal potential of Ca^{2+} current indicate, nevertheless, that Ca^{2+} channels have an extremely low permeability, if any, to Cs^+ or K^+. The present technique also allows measurement of the sensitivity of Ca^{2+} channel current to changes in external Na^+ or K^+ concentration while avoiding secondary changes in intracellular ionic conditions. The results suggest that Na^+ do not normally contribute significant charge to the Ca^{2+} current and indicate that K^+ have no direct effect on the Ca^{2+} channel.

METHODS

Guinea-pig single ventricular cells were obtained by enzymatic dissociation as described previously (Matsuda, Noma, Kurachi & Irisawa, 1982).

Electrodes. Patch electrodes (inner tip diameter $\sim$ 4 μm) were fabricated from capillaries of high melting temperature glass with no glass fibres inside. After heat polishing the tip of the electrode, it was filled with internal solution, the tip by capillarity and the shank via a syringe. The electrode resistance ranged between 1 and 2 MΩ.

Solutions. The compositions of the main external and internal solutions are listed in Table 1. To attain a high seal resistance with ease, we first filled the patch electrode with a solution containing 1 mM-$CaCl_2$. After making the seal, but before the cell membrane was ruptured, the pipette solution

was changed to one containing 5 mM-ethyleneglycol-bis(β-aminoethylether)*N,N'*-tetraacetic acid (EGTA) to keep the intracellular Ca^{2+} concentration at a low level. For details of the technique for exchanging the solution inside patch electrodes see Soejima & Noma (1984). After rupture of the membrane, all pipette (i.e. internal) solutions also contained 5 mM-adenosine 5'-triphosphoric acid (ATP as dipotassium salt; Sigma) to diminish 'run-down' of the Ca^{2+} current (Irisawa & Kokubun, 1983). The pH of external solutions was adjusted to 7·4 by HEPES–NaOH buffer or Tris buffer. In internal solutions, pH was adjusted to 7·3 by 5 mM-HEPES–KOH or HEPES–CsOH. The temperature of the solution in the chamber was kept at 36–37 °C.

TABLE 1. Composition of solutions (mM)

External

	NaCl	NaH_2PO_4	KCl	$CaCl_2$	$MgCl_2$	Glucose	HEPES–NaOH	Tris Cl	V_j (mV)
Normal solution	144	0·33	5·4	1·8	0·53	5·5	5	—	0
	149	0·33	5·4	1·8	0·53	5·5	—	5	0
0 Na^+, 0 K^+ solution	—	—	—	1·8	0·53	5·5	—	149	2

Internal

	K aspartate	KCl	Cs aspartate	CsCl	EGTA	ATP*	HEPES	V_j (mV)
K^+-rich solution	110	20	—	—	5	5	5	−13
Cs^+-rich solution	—	—	110	20	5	5	5	−13

The pH of external solutions was adjusted to 7·4.

In internal solutions, pH was adjusted to 7·3 by 5 mM-HEPES–KOH or HEPES–CsOH.

V_j was the liquid junction potential between the solution and the normal external solution; the sign was positive when the normal solution side was negative.

* ATP (dipotassium salt).

The liquid junction potentials between the normal external solution and each of the other solutions were directly measured, assuming the junction potentials at the tip of a 3 M-KCl electrode in these solutions to be negligible. The values are listed in Table 1; the sign was positive when the normal solution side was negative. Corrections of the membrane potentials were then made as described by Hagiwara & Ohmori (1982).

Recording techniques. Our voltage-clamp circuit for use with single pipettes is largely as described by Hamill *et al.* (1981) except that the feed-back resistor in the current–voltage converter is 100 MΩ and command potentials are fed to the positive input. The series resistance arising mainly at the patch-electrode tip was compensated by summing a fraction of the converted current signal to the command potential: for details see Ohara, Kameyama, Noma & Irisawa (1983). When the tip of the patch electrode was in the normal external solution, the command d.c. potential was adjusted to make the current through the patch electrode zero and this voltage was taken as the reference potential. The resistance of the electrode was continuously monitored by observing the current produced by a 1 mV voltage pulse. After the pipette tip was pressed against the cell surface, a negative pressure of 30–50 cmH_2O was applied to the interior of the electrode. Within 10–30 s a tight seal between the electrode tip and the cell membrane (10–100 GΩ) was usually established. While maintaining the negative pressure, the internal solution containing 1 mM-$CaCl_2$ was replaced by the low-Ca^{2+} solution. Then the cell membrane at the tip of the electrode was ruptured by applying further brief suction. Rupture was indicated by a large increase of the capacitive currents. Voltage steps were applied from a holding potential of about −45 mV every 6 s. Current and voltage signals were stored on magnetic tape (TEAC, R-210) for later computer analysis (HITACHI, E-600).

Membrane capacitance determined in K^+-, Na^+-free solutions from the ratio of the area under the capacitive current transient and the magnitude of the voltage step (10 mV hyperpolarization from a holding potential of −45 mV) was 132±41 pF (mean±S.D., $n = 12$). Specific membrane

capacitance was calculated by dividing total membrane capacitance by the surface area of the cell. Assuming ventricular cells to be a simple rectangular block with thickness of 6 μM (Powell, Terrar & Twist, 1980), the calculated surface areas range from 4800 to 7200 μm^2, and the resulting specific membrane capacitance is $2{\cdot}33 \pm 0{\cdot}71$ $\mu F/cm^2$ ($n = 12$). If T-system and surface caveolae increase the total membrane surface area by 78% (Severs, Slade, Powell, Twist & Warren, 1982), then the specific membrane capacitance is $\sim 1{\cdot}3$ $\mu F/cm^2$, a value within the accepted range of most biological membranes (Cole, 1968).

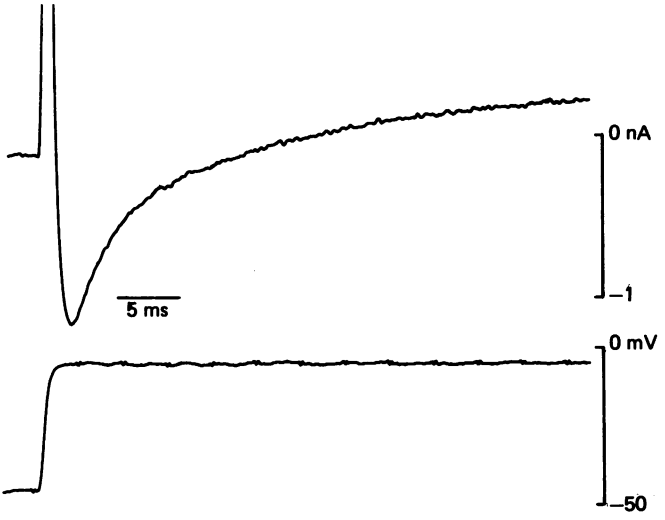

Fig. 1. Records of membrane current (upper trace) and membrane potential (lower trace) under the voltage clamp using a single patch-clamp electrode (1·6 MΩ resistance). The membrane potential was recorded by an independent micro-electrode filled with 3 M-KCl (15 MΩ). Command pulse from −45 to −5 mV was applied. The records were reproduced from the magnetic tape which has the flat frequency response up to 1·25 kHz. The peak of the capacitive current is off scale. Na^+-, K^+-free external solution; Cs^+-rich internal solution.

To assess the adequacy of voltage control immediately after start of a clamp step, a settling time was measured by introducing an independent intracellular micro-electrode (Fig. 1). The intracellular potential (lower trace) recorded at a distance of 30 μm from the patch electrode followed the command with a short delay and reached a steady level within 1·5 ms, whereas the peak of the Ca^{2+} current occurred only after 2·5 ms (upper trace). The settling of the membrane potential is slow enough to preclude resolution of activation kinetics, but not to interfere with measurement of the peak amplitude and the inactivation time course of the Ca^{2+} current.

Average results throughout this paper are given as mean ± S.D.

RESULTS

Effects of K^+ and Na^+ removal on the steady-state currents

Altering ionic composition on both sides of the membrane offers a direct approach to characterizing the background current while minimizing its interference with analysis of inward Ca^{2+} current. To eliminate currents carried by K^+, we first replaced intracellular K^+ with Cs^+, and then removed extracellular K^+. Fig. 2 shows the current changes caused by replacing the K^+-rich solution inside the electrode with the Cs^+-rich one. After the Cs^+-rich solution arrived at the tip of the electrode

(arrowhead), the holding current began to shift inwards within 1 min and reached a new steady level within 3 min. The currents recorded during 300 ms depolarizing pulses from −43 to −3 mV revealed loss of the negative slope in the steady-state membrane current–voltage (*I–V*) curve. The time-dependent current deflexion during the clamp pulse was slightly decreased and an increasing inward current appeared on repolarization. The inward shift of the holding current was observed in every cell examined.

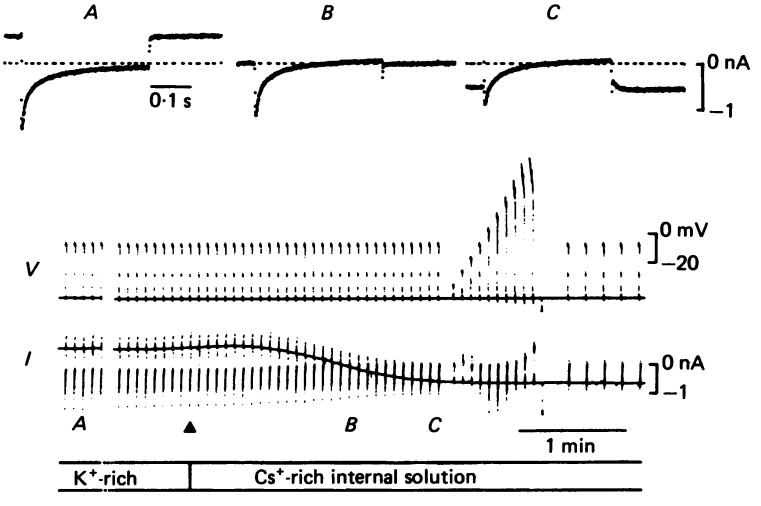

Fig. 2. Current changes accompanying replacement of the K^+-rich solution inside the electrode with the Cs^+-rich one. Records of membrane potential (*V*) and current (*I*) are continuous except for omission of a 2 min segment as indicated. The 300 ms depolarizing pulses to −3 mV from a holding potential of −43 mV were applied every 5 s. Arrowhead indicates estimated time of arrival of the Cs^+-rich solution at the tip of the electrode determined in other, control, experiments using dye solution. Current records in the upper panel correspond to times indicated by *A*, *B* and *C* in the lower panel. After recording *C*, different clamp pulses were applied. Normal external solution (Tris buffer).

I–V relations measured at the end of 300 ms pulses before (open circles) and after (filled circles) exchanging the K^+-rich internal solution for Cs^+-rich one are shown in Fig. 3. Perfusion of the pipette with Cs^+-rich solution caused virtual disappearance of the negative slope, a shift of the zero-current potential from −80 to −33 mV, and an increase in outward current between −30 and +30 mV. The large inward currents, which persisted after pipette perfusion with Cs^+-rich solution, were eliminated by removing extracellular K^+ (filled triangles) and the *I–V* curve became almost linear except at large positive potentials ($\geqq$ +40 mV). Removal of extracellular Na^+ as well as K^+ (open triangles) had little further effect on the *I–V* relations. Thus, it is evident that removal of K^+ from both sides of the membrane eliminates virtually all background current except at large positive potentials. In the voltage range from −25 to +25 mV, a slight inward deflexion was observed. This might be attributed to a non-inactivating component of the Ca^{2+} channel current. The amplitude of this component varied among cells (cf. Figs. 6, 7, 8, 9 and 11).

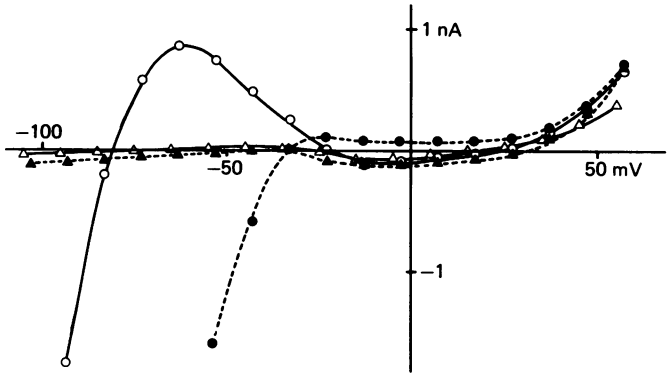

Fig. 3. *I–V* relations measured at the end of the 300 ms pulses in the presence of various internal and external solutions; with K^+-rich internal and normal external (Tris buffer) solutions (open circles); Cs^+-rich internal and normal external (Tris buffer) solutions (filled circles); Cs^+-rich internal and K^+-free external solutions (filled triangles); Cs^+-rich internal and Na^+-, K^+-free external solutions (open triangles). Holding potential is −43 mV in the presence of external Na^+ and −45 mV in the absence of Na^+. The same cell as in Fig. 2.

TABLE 2. Resting membrane conductances* ($\mu S/cm^2$) in the presence of various internal and external solutions

	K^+-rich/NES	Cs^+-rich/NES	Cs^+-rich/0 K^+	Cs^+-rich/0 K^+, 0 Na^+
Group I ($n = 10$)	1345±545	705±322		
		51.3±9.2%†		
Group II ($n = 9$)		780±340	15.5±4.8	
			2.4±1.6%†	
Group III ($n = 12$)		660±195		11.8±5.7
				1.9±1.1%†

* The total surface membrane area including T-system and surface caveolae was used for calculation.

† Average of the percentage remaining of the original membrane conductance which was obtained in each cell.

NES: normal external solution (HEPES–NaOH).

0 K^+ solution was made simply by omitting 5·4 mM-KCl from the normal external solution.

The resting input resistance was measured as the limiting slope of the *I–V* curve at negative potentials. Input resistance so defined increased from 6·7 MΩ in the normal solution to 10·0 MΩ during perfusion of the pipette with Cs^+-rich solution. The input resistance was further increased to 550 MΩ and to 1·0 GΩ, respectively, by removing external K^+, and by replacing external Na^+ of the K^+-free solution with $Tris^+$. Table 2 summarizes the effects on resting conductances of altering the ionic milieu on both sides of the cell membrane. After replacing internal K^+ with Cs^+, subsequent removal of external K^+, or removal of both external Na^+ and K^+, reduced

resting membrane conductance to about 1 % of the control value obtained with the normal external and internal solutions. This suggests that, at negative potentials, some 99 % of resting membrane slope conductance is attributable to K^+ and the contribution from Na^+ is negligibly small. It is also suggested that the membrane conductance to Cl^-, which is the only conducting ion in the Na^+-, K^+-free solution, is trivial.

The effectiveness of the replacement of intracellular K^+ with Cs^+ on switching the solution perfusing the pipette can be roughly estimated. On the basis of the above conductance measurements, the zero-current potential can be considered approximately equal to the equilibrium potential for K^+ (E_K). The zero-current potentials for I–V curves obtained in the normal external solution with either K^+-rich solution, or Cs^+-rich solution, inside the pipettes were $-80{\cdot}0 \pm 1{\cdot}7$ mV ($n = 10$), and $-31{\cdot}14 \pm 7{\cdot}6$ mV ($n = 58$), respectively. These values suggest that the intracellular K^+ concentration fell from 110 mM to an average of 18 mM during perfusion of the pipettes with Cs^+-rich solution. The latter value is quite reasonable considering both the small tip diameter of the patch electrodes (i.e. inner tip diameter $\sim 4\ \mu$m) and the fact that the Cs^+-rich solution itself contained 10 mM-K^+.

Time-dependent outward current induced by replacing internal K^+ with Cs^+

After exchanging the K^+-rich internal solution for the Cs^+-rich one, the peak inward current deflexion during the depolarizing pulse gradually decreased, a result that might be interpreted as a reduction of the Ca^{2+} current (Fig. 2). This change, however, is most probably due to interference from an overlapping outward current induced by lowering the internal K^+ concentration. The existence of such an outward current, over the entire voltage range usually used for studying Ca^{2+} currents, is revealed by blocking the Ca^{2+} channel with 0·9 mM-Co^{2+} as illustrated in Fig. 4. In the absence of Ca^{2+} current, a decaying outward current follows the positive spike of capacitive current during depolarizing pulses, and the time course of current decay is accelerated with stronger depolarization. An increasing inward current is observed on repolarization to the holding potential. The presence of this time-dependent current with Cs^+-rich internal solution, and its absence with K^+-rich internal solution, can be demonstrated directly (Fig. 5*A*) by exchanging the pipette solution while exposing the cell to 0·9 mM-Co^{2+} to block Ca^{2+} currents.

The time-dependent current that remains after suppression of Ca^{2+} channel current is presumably carried by K^+. As mentioned above, E_K is approximately -31 mV in cells dialysed with the Cs^+-rich solution. Because K^+ current must be outward at voltages positive to that level, the current decay observed in Co^{2+}-treated cells during depolarizing clamp pulses is likely to reflect a time-dependent decrease of membrane K^+ conductance, and the increasing inward current on repolarization to potentials negative to -31 mV a time-dependent increase of K^+ conductance. This suggestion is supported by the observation that, after blocking the Ca^{2+} current with 0·9 mM-Co^{2+}, both the decaying outward current on depolarization and the increasing inward current on repolarization were abolished by addition of 0·2 mM-Ba^{2+} to the external solution (Fig. 5*B*). Such low concentrations of Ba^{2+} are well known to suppress K^+ currents, especially currents flowing through inwardly rectifying K^+ channels (Sakmann & Trube, 1984*b*).

That this K^+ current revealed in Figs. 4 and 5 does indeed flow via inward rectifier

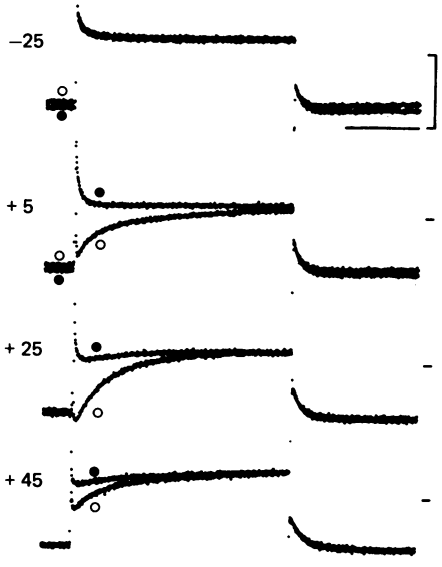

Fig. 4. Currents obtained before (open circles) and after (filled circles) Co^{2+} application. The numbers to the left of each current trace refer to the potential levels during the voltage-clamp steps (in mV). Holding potential, −45 mV; Cs^+-rich internal solution. In 5·4 mM-K^+, Na^+-free external solution, 0·9 mM-Co^{2+} was substituted for an equimolar amount of Ca^{2+}. The marks to the right of each record indicate zero current. The time calibration indicates 100 ms and the current calibration represents 1 nA.

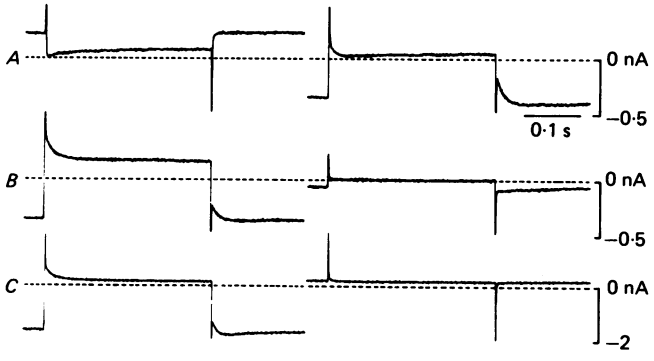

Fig. 5. Identification of the outward decaying current on depolarization and increasing inward current on repolarization induced by lowering the intracellular K^+ concentration. *A*, induction of the time-dependent current by replacing the K^+-rich internal solution (left) with the Cs^+-rich one (right) in Na^+-free external solution containing 5·4 mM-K^+, 0·9 mM-Co^{2+} and 0·9 mM-Ca^{2+}. 300 ms step depolarizations from −45 to −5 mV. *B*, suppression of the time-dependent current by Ba^{2+}. Current records before (left) and after (right) addition of 0·2 mM-Ba^{2+} to Na^+-free external solution containing 5·4 mM-K^+, 0·9 mM-Co^{2+} and 0·9 mM-Ca^{2+}. Cs^+-rich internal solution. 300 ms depolarizations from −45 to −25 mV. *C*, suppression of the time-dependent current by K^+-free solution. Current records with Cs^+-rich internal solution in Na^+-free external solution containing 5·4 mM-K^+ (left) and 0 K^+ (right). 300 ms depolarizations from −45 to −25 mV.

K^+ channels is strongly suggested by the disappearance of both inward and outward time-dependent currents on removal of extracellular K^+, as illustrated in Fig. 5*C*. Abolition of the inwardly rectifying component of the steady-state membrane *I–V* relationship by removal of external K^+ has already been shown in Fig. 3 (compare filled triangles with filled circles); the concomitant disappearance of the time-dependent current (Fig. 5*C*) argues strongly that the steady-state and time-dependent currents both flow in inward rectifier channels.

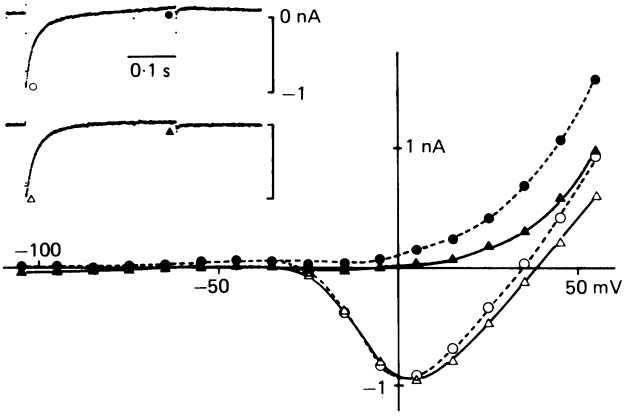

Fig. 6. Current changes accompanying replacement of intracellular K^+ with Cs^+ in Na^+-, K^+-free external solution. Currents elicited by voltage-clamp steps in 10 mV increments from −45 mV are plotted against the pulse potential before (open circles, peak; filled circles, 300 ms) and after (open triangles, peak; filled triangles, 300 ms) K^+ replacement. Current traces correspond to depolarizing pulse to +5 mV; compare these with records *A* and *C* in Fig. 2.

If the induction of the overlapping outward current transient by replacement of intracellular K^+ with Cs^+ shown in Fig. 5*A* is responsible for the apparent decrease of the peak Ca^{2+} current shown in Fig. 2, then no decrease of peak inward current should occur on replacing cellular K^+ with Cs^+ after suppressing the inwardly rectifying K^+ current by removal of external K^+. The steady-state *I–V* curves in Fig. 6 confirm that inwardly rectifying K^+ current is suppressed in K^+-free solution both with K^+-rich pipette solution and with Cs^+-rich pipette solution. But, under these conditions, although reduction of the internal K^+ concentration caused some decrease in the late outward current, there was no decrease in the peak, net inward currents (Fig. 6, inset). The decrease in the late current is attributed to suppression of the delayed rectifying K^+ current, which develops during the strong depolarizing pulses in the presence of intracellular K^+.

Effect of external K^+ on the Ca^{2+} channel current

Related to the above results, we found that removal of extracellular K^+ with the Cs^+-rich solution inside the electrode led to a greater inward current deflexion during the depolarizing pulse (Fig. 7), as if the Ca^{2+} current were increased by lowering external K^+ concentration. This change was accompanied by a reduction of the

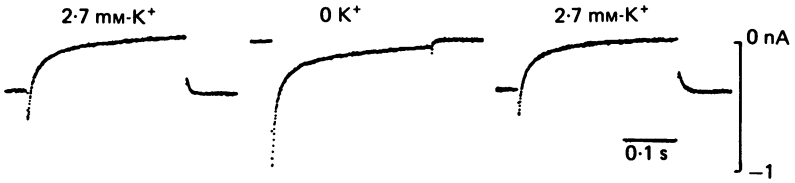

Fig. 7. Apparent increase in Ca^{2+} current induced by reduction of external K^+. Cs^+-rich internal solution; 300 ms depolarizations from −45 to +15 mV. K^+ concentration of Na^+-free external solution was reduced from 2·7 mM (left) to 0 (middle), and returned to 2·7 mM (right).

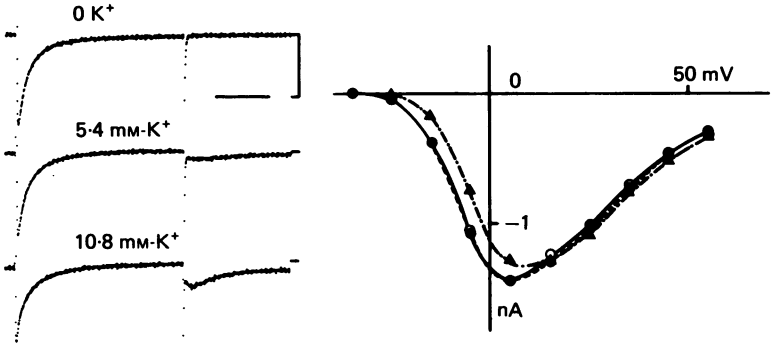

Fig. 8. Ca^{2+} current was little affected by external K^+ in the presence of 0·2 mM-external Ba^{2+}. Current records were taken during voltage pulses from a holding potential of −45 to +5 mV. Calibrations are the same as in Fig. 4. The difference in current between the peak and the steady-state level at the pulse potential is plotted against the membrane potential in 0 K^+ (open circles), 5·4 mM-K^+ (filled circles) and 10·8 mM-K^+ (filled triangles) external solution without Na^+. Cs^+-rich internal solution.

inward holding current, and both effects reversed completely on returning to the control K^+ concentration.

To see whether the overlapping outward current described in the preceding section might be responsible for such an apparent, K^+-dependent, change in the Ca^{2+} current, we examined the effect of varying the external K^+ concentration after suppressing inwardly rectifying K^+ current with Ba^{2+} (Fig. 8). The cell was superfused with Na^+-free external solutions containing 0·2 mM-Ba^{2+} and different K^+ concentrations (0, 5·4 and 10·8 mM). That the holding current level in all three solutions was close to zero suggests that the inward rectifier K^+ channel was substantially blocked by 0·2 mM-Ba^{2+} (see also Fig. 5*B*). The amplitudes of the Ca^{2+} channel currents were measured as the difference between the peak and the steady-state current levels at the pulse potential and are plotted against the pulse potential in the right panel. Under these conditions, reduction of external K^+ concentration from 5·4 (filled circles) to 0 mM (open circles) had no obvious effect on the Ca^{2+} current. Elevation of external K^+ concentration from 5·4 to 10·8 mM (filled triangles) caused a slight decrease in amplitude of the Ca^{2+} current, which might be attributable to persisting interference from the residual inwardly rectifying K^+ current in the 10·8 mM-K^+ solution. Essentially similar results were obtained with Ca^{2+}-free external solutions containing

0·9 mM-Ba^{2+}. In this case, the Ba^{2+} current through the Ca^{2+} channel remained constant at different K^+ concentrations from 0 to 10·8 mM (not illustrated). These findings exclude any significant, direct, inhibitory action of K^+ on the Ca^{2+} channel, and strongly suggest that apparent effects of extracellular K^+ are mediated by changes in the size of an overlapping K^+ current.

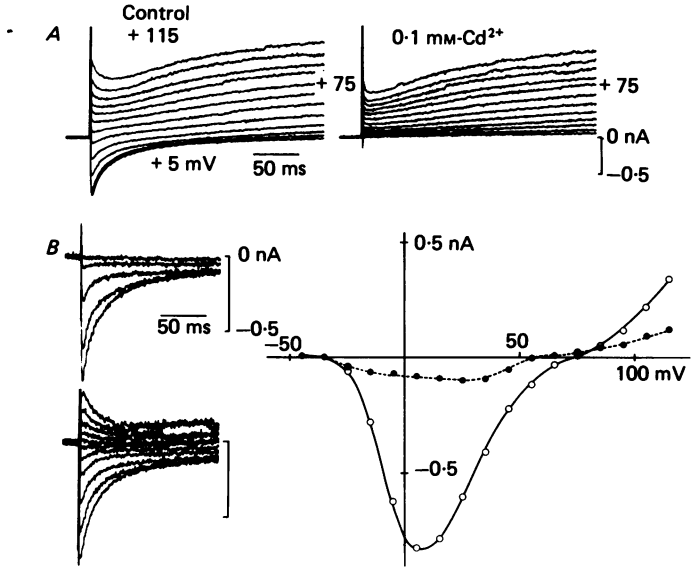

Fig. 9. Isolation of Ca^{2+} current. *A*, currents measured before (left) and after (right) addition of 0·1 mM-Cd^{2+}. Na^+-, K^+-free external solution; Cs^+-rich internal solution containing 20 mM-TEA Cl in place of CsCl. Superposed current records were elicited by 300 ms clamp depolarizations from +5 to +115 mV in 10 mV increments. Holding potential, −45 mV. *B*, difference between currents before and after Cd^{2+} addition. In the left column, upper series correspond to depolarizations from −35 to +5 mV and lower series correspond to depolarizations from +15 to +115 mV. Difference currents (open circles, peak; filled circles, 300 ms) are plotted against the membrane potential in the graph at the right.

Reversal of Ca^{2+} channel current

The results presented so far demonstrate that the major components of both time-dependent and steady-state 'background' currents can be largely abolished by using Cs^+-rich, low-K^+ pipette solution, and by superfusing the cell with K^+-free, Na^+-free external solution. We therefore used these conditions in our attempts to further isolate Ca^{2+} channel current. In theory, the Ca^{2+} channel current could be isolated from the background currents remaining in Na^+-, K^+-free external solution with Cs^+-rich internal solution if some agent were available which selectively suppressed the Ca^{2+} channel current. We tried this approach to isolating the Ca^{2+} channel current by subtracting from the control current the current obtained after application of an inorganic Ca^{2+} blocker. Fig. 9*A* illustrates a typical series of current records obtained before (left) and after (right) addition of 0·1 mM-Cd^{2+} to the external

solution. Although the background currents were greatly reduced by removing K^+ from both sides of the membrane, by using Na^+-free external solution, and by applying tetraethylammonium (TEA) intracellularly, the control records show a relatively large, slowly-increasing, time-dependent outward current at potentials above +30 mV. At potentials greater than +70 mV, a more rapidly decaying outward current followed the positive spike of capacitive current. Since the pipette solution contained 5 mM-EGTA, the intracellular Ca^{2+} concentration was presumably too small to cause any significant outward Ca^{2+} current, so that the outward current was most likely carried by Cs^+. At the concentration used, Cd^{2+} blocks inward Ca^{2+} current almost completely, and also removes some outward current at large positive potentials, but appears to have little effect on the background current at the negative potential ranges (not illustrated). Essentially the same result was obtained in ten cells. After washing out the Cd^{2+}, the inward Ca^{2+} current recovered in all ten cells, though incompletely in most cells, while the outward currents remained depressed in three of the ten cells.

The left column of Fig. 9*B* shows the difference between the currents before and after Cd^{2+} addition (Cd^{2+}-sensitive currents). In the graph at the right, the peak amplitude (open circles) and amplitude near the end of the 300 ms clamp pulse (filled circles) of the Cd^{2+}-sensitive currents have been plotted against the membrane potential. The initial current peaks between 0 and +10 mV, at a magnitude of 0·8 nA, and reverses polarity at +75 mV. On the other hand, the late current reverses at +55 mV. A reversal potential for the late current 10–20 mV less positive than that for the initial current was observed in all ten cells in which the effects of Cd^{2+} were evaluated. Moreover, both initial and steady-state *I–V* relations for the Cd^{2+}-sensitive current at large positive potentials are markedly non-linear, with conductance increasing on either side of an inflexion near the reversal potential. These findings are all consistent with a view derived from experiments on *Limnea* neurones that the Cd^{2+}-sensitive current includes at least two components; a Ca^{2+} channel current and a relatively non-specific outward current (Byerly & Hagiwara, 1982).

Several other findings suggest such an interpretation. Thus, the Cd^{2+}-sensitive outward current at large positive potentials might be considered separable into time-dependent and time-independent components. However, the time-dependent outward current component and the inward Ca^{2+} current seen at less positive potentials do not seem to be well correlated. For example, the decaying outward current did not recover from the Cd^{2+} application to the same degree as the inward Ca^{2+} current did (not illustrated). In addition, no consistent pattern was observed between different cells, as illustrated in Fig. 10*A*: one cell has a small inward current and a large time-dependent outward current component (left), while another cell has a large inward current but a small time-dependent outward component (right). Furthermore, substitution of 0·9 mM-Co^{2+} for half of the external Ca^{2+} suppressed the inward current without much effect on the time-dependent outward component (Fig. 10*B*). Decaying outward current still present after a blocking dose of nifedipine was observed by other workers (Mitchell, Powell, Terrar & Twist, 1983). These results suggest that the decaying outward component of Cd^{2+}-sensitive current is carried not through Ca^{2+} channels but via some other voltage-dependent conductance.

The time-independent current component of the drug-sensitive current was

revealed by Co^{2+} application as well as by Cd^{2+} (e.g. Fig. 10*B*). This time-independent component also seems unlikely to be carried through Ca^{2+} channels because, while inward Ca^{2+} current was about 90% inactivated by a 100 ms conditioning pre-pulse to +15 mV (dotted trace), the outward current elicited by a large positive pulse was little affected (Fig. 10*C*). These findings led us to conclude that a major part of the

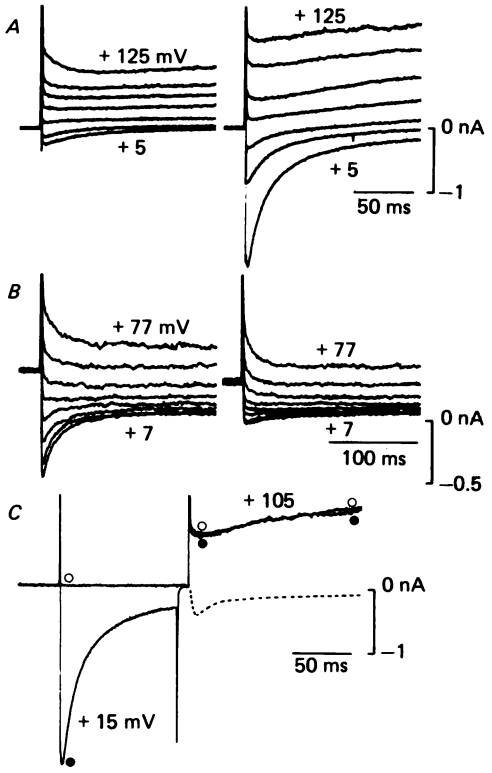

Fig. 10. *A*, current records taken from two different cells. Na^+-, K^+-free external solution with 5 mM-4-aminopyridine (4-AP); the same internal solution as in Fig. 9. In each cell, superposed currents were elicited by voltage-clamp depolarizations from +5 to +125 mV in 20 mV increments. Holding potential, −45 mV. *B*, currents before (left) and after (right) substitution of 0·9 mM-Co^{2+} for an equimolar amount of Ca^{2+}. Normal external solution (HEPES buffer); K^+-rich internal solution. Superposed current records were elicited by voltage-clamp depolarizations from +7 to +77 mV in 10 mV increments. Holding potentials, −43 mV. *C*, effects of depolarizing pre-pulse. Current records elicited by the test-clamp step to +105 mV with (filled circles) or without (open circles) the pre-pulse to +15 mV are superposed. Dotted trace indicates the current in response to the test-clamp step to +15 mV preceded by the conditioning pulse of the same amplitude. Holding potential, −45 mV; the same internal and external solutions as in Fig. 9.

outward current at large positive potentials observed in our experimental conditions is due to some conductance system other than the Ca^{2+} channels and, therefore, that the reversal potential of Ca^{2+} blocker-sensitive current is not the reversal potential of genuine Ca^{2+} channel current.

On the other hand, we presume that the Cd^{2+}-sensitive currents shown in Fig. 9*B*

at lower potentials, i.e. up to about +35 mV, are predominantly pure Ca^{2+} currents. The decay of Ca^{2+} current isolated in this way can be well described by the sum of two exponentials with time constants at +5 mV, 52 ± 11 ms, and 8 ± 3 ms, respectively ($n = 10$). These data compare reasonably well with previously published time constants for decay of Ca^{2+} channel current in isolated ventricular cells (Isenberg & Klöckner, 1982*b*; Irisawa & Kokubun, 1983).

Effects of Na^+ removal on the Ca^{2+} channel current

As described above, the persistence of a relatively non-specific outward current which was activated at large positive potentials and which showed some sensitivity to Ca^{2+} channel blockers, hampered determination of the reversal potential for Ca^{2+} channel current. We therefore used more negative potentials, where the contaminating non-specific outward current is negligibly small, in order to assess the selectivity of Ca^{2+} channels with respect to Na^+ from measurements of the sensitivity of Ca^{2+} channel current to a reduction of external Na^+ concentration.

Such an approach has been taken in multicellular preparations, and observations that reduction of extracellular Na^+ decreased the peak amplitude of the Ca^{2+} channel current led to the suggestion that some fraction of the Ca^{2+} channel current may be carried by Na^+ (Lenfant, Mironneau & Aka, 1972 (cited by Coraboeuf, 1980); Reuter & Scholz, 1977; Noma, Yanagihara & Irisawa, 1977; for review see Coraboeuf, 1980). There is the possibility, however, that Na^+-poor solutions decrease Ca^{2+} extrusion via the Na^+–Ca^{2+} exchange mechanism, and that the resulting increase in intracellular Ca^{2+} concentration might reduce Ca^{2+} channel current (Kokubun & Irisawa, 1984). Such a depressant effect of raised intracellular Ca^{2+} has been observed in a variety of neurones (Kostyuk & Krishtal, 1977; Akaike, Lee & Brown, 1978; Plant, Standen & Ward, 1983). Thus, it is essential to study the effect of lowering Na^+ on the Ca^{2+} channel current while controlling the level of intracellular Ca^{2+}.

Fig. 11 illustrates typical results from one of these experiments. The upper panel shows current records elicited by 300 ms depolarizing voltage steps of 50 mV, in K^+-free solution, in the presence of 3·1 μM-tetrodotoxin (TTX; Sankyo). The left trace was obtained in the presence of 149 mM-Na^+ and the one at the right after substitution of $Tris^+$ for all of the Na^+. The holding potential was kept constant, at −43 mV, despite the expected, −2 mV change of the junction potential in the Na^+-free solution. Semilogarithmic plots of the decaying phases of the inward current revealed that removal of external Na^+ had no effect on the time course of the Ca^{2+} channel current. In the total of six experiments, the inactivation time constant in the presence and the absence of Na^+ averaged 9 ± 2 ms *vs.* 8 ± 2 ms for the fast component, and 50 ± 13 ms *vs.* 52 ± 14 ms for the slow component.

The difference in current between the peak and the steady-state level at the pulse potential is plotted against membrane potential in the lower panel. The curves obtained in the presence (open circles) and in the absence (open triangles) of external Na^+ are almost exactly superimposable. In six experiments, the amplitude of the Ca^{2+} channel current after Na^+ removal averaged 99·5 ± 7·0 % of the control value. Thus, under the conditions of our experiments, complete removal of external Na^+ caused no significant decrease of the Ca^{2+} channel current. This result indicates that the contribution of Na^+ influx to the Ca^{2+} channel current is negligibly small in guinea-pig

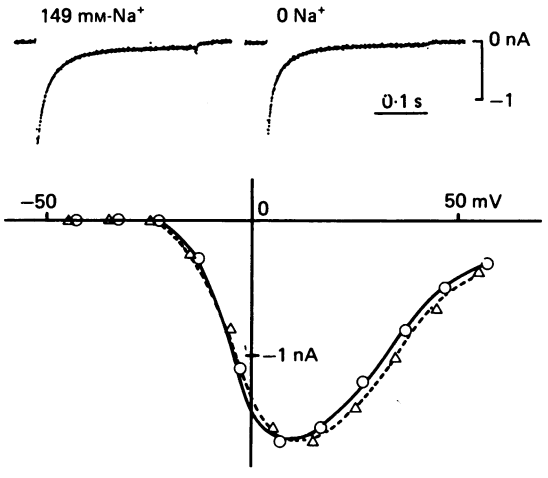

Fig. 11. Effects of Na^+ removal on Ca^{2+} current. Current records correspond to the 300 ms depolarizing step of 50 mV. Holding potential is −43 mV (left) and −45 mV (right). The fast inactivation time constant is 13 ms and the slow time constant is 55 ms in the presence of Na^+, while they are 13 and 56 ms, respectively, in the absence of Na^+. K^+-free external solution with 3·1 μM-TTX; Cs^+-rich internal solution. The difference in current between the peak and the steady-state level at the pulse potential was plotted against the membrane potential with (open circles) and without (open triangles) Na^+.

ventricular cells. In other words, at normal extracellular Ca^{2+} concentrations, Ca^{2+} channel current is carried almost exclusively by Ca^{2+}.

DISCUSSION

Efficiency of internal dialysis using patch-clamp electrode

Although a patch-clamp electrode used in our study has smaller internal tip diameter than a conventional suction electrode (i.e. ~ 4 μm as against 8–10 μm; cf. Irisawa & Kokubun, 1983), the restricted aims of internal dialysis for the purposes of this study appear to have been largely attained, namely (1) replacing intracellular K^+ with Cs^+ and (2) maintaining the intracellular Ca^{2+} concentration at a low level. Although intracellular K^+ concentration was not measured directly in our preparations, it seems likely that K^+ replacement was rather substantial. In spite of the fact that steady-state inward Na^+ currents appear to be very small in these guinea-pig ventricular cells (e.g. Fig. 3), our rough estimate of 18 mM for the intracellular K^+ concentration should be considered a minimum estimate, in so far as the zero-current potential will approximate E_K only in the absence of all inward current components, passive or active, carried by cations or anions. Nevertheless, it seems likely that the cellular K^+ concentration will have approached more closely that of the internal solution, i.e. 10 mM, during exposure to the K^+-free solution when inward K^+ currents were abolished. The effectiveness of the Ca^{2+} buffering was indicated by the absence of contractile activity even at strong depolarization and, in the experiments done in Na^+-containing solution (e.g. Figs. 3 and 11), absence of

the oscillatory inward current usually induced in the K^+-free solution (Eisner & Lederer, 1979; Matsuda *et al.* 1982).

With regard to Ca^{2+} channels, the phenomenon of run-down of the Ca^{2+} current with time has been attributed to wash-out of soluble intracellular molecules, because the Ca^{2+} channel current has been found to decay more rapidly when the rate of exchanging intracellular ions was increased (Byerly & Hagiwara, 1982). As previously reported by Irisawa & Kokubun (1983), ATP in the internal solution caused a halt of the decay of the Ca^{2+} current and of decrease in the membrane resistance.

Time-dependent K^+ current overlapping the Ca^{2+} channel current

It has previously been suggested that K^+ removal increases the Ca^{2+} channel current (Goto, Tsuda & Yatani, 1977; Tsuda, 1979; Lederer & Eisner, 1982), and it has been shown that net inward current decreases in high-K^+ solution (Noma, 1976). These effects of K^+ have been explained by assuming a direct inhibitory action of external K^+ on the Ca^{2+} channels (Tsuda, 1979) or by a change in intracellular ion concentrations mediated by changes in Na^+–K^+ pump activity (Lederer & Eisner, 1982). The present results, obtained while maintaining the intracellular ionic composition, rule out any direct action of external K^+ on the Ca^{2+} channel and provide a possible alternative explanation for some of the above-mentioned effects of K^+ on net current in terms of changes in the size of a transient K^+ current that overlaps the Ca^{2+} current.

Studying the effect of K^+ on the Ca^{2+} current, we noticed a peculiar decaying outward current activated at potentials more positive than -25 mV in 5·4 mM-external K^+ solution and with Cs^+-rich solution in the pipette. Overlapping the Ca^{2+} current, it caused an apparent decrease in the net inward current. As already argued, the voltage dependence of this current, i.e. decaying outward current positive to the expected level of E_K and increasing inward current negative to E_K (Figs. 4 and 5), together with its abolition by 0·2 mM-Ba^{2+} (Fig. 5*B*), indicate that it is carried by K^+. Furthermore, that current is seen only in our experiments under conditions in which the steady-state *I–V* curve shows the marked inward rectification which is characteristic of background inwardly rectifying K^+ channels in cardiac and skeletal muscle (e.g. Katz, 1949; Adrian & Freygang, 1962; Hall, Hutter & Noble, 1963). It disappears (Fig. 5*B* and *C*) when inward rectification of the steady-state *I–V* curve is abolished by removal of external K^+ (cf. Fig. 3) or by exposure to low concentrations of Ba^{2+} (not illustrated). Similar abolition of K^+ currents in inwardly rectifying K^+ channels by exposure to K^+-free solution has been demonstrated previously, for example in isolated cells from bovine ventricle (Isenberg & Klöckner, 1982*a*) and in tunicate egg cells (Ohmori, 1978). Thus, our findings strongly suggest that this time-dependent current flows in inwardly rectifying K^+ channels, and reflects the time course of gating of those channels.

The well-known dependence of inwardly rectifying K^+ conductance on the driving force for K^+ movement across the cell membrane (e.g. Hodgkin & Horowicz, 1959) provides a possible explanation for the appearance of the time-dependent K^+ current on reducing intracellular K^+ concentration. Thus, with K^+-rich pipette solution, the usual holding potential of -45 mV lies some 40 mV positive to E_K. Under these conditions, inwardly rectifying K^+ channels should be mostly closed in the steady

state, and depolarization should lead only to further closing, so that voltage pulses between −45 and 0 mV are not expected to be associated with much change in K^+ current. However, when intracellular K^+ concentration is lowered by substitution with Cs^+, E_K is shifted to about −30 mV and, since the K^+ driving force is then inward, inwardly rectifying K^+ channels are expected to be open. Depolarization to voltages positive to E_K should then lead to channel closure and repolarization to channel reopening, events presumably causing changes in membrane K^+ current. If this explanation is correct, then similar time-dependent changes in K^+ current to those shown in Figs. 4 and 5, may be expected to occur also in multicellular preparations whenever voltage steps are made within range of potentials in which the conductance of inwardly rectifying K^+ channels changes substantially; e.g. depolarizing voltage steps from a holding potential within 20–30 mV of E_K (Sakmann & Trube, 1984*a*). This problem is expected to be greater when more negative holding potentials are used, or when E_K is lowered by raising extracellular K^+ concentration or by K^+ accumulation in intercellular clefts.

Isolation of the Ca^{2+} channel current

When internal K^+ is replaced with Cs^+ and external K^+ and Na^+ are removed, sizeable time-dependent outward currents remain at large positive potentials. Since the internal Ca^{2+} concentration can be assumed to be too small for Ca^{2+} to carry any significant outward current, this remaining current is most likely carried by Cs^+. We found that the outward currents were always changed (usually reduced) when the Ca^{2+} current was suppressed by the inorganic blockers such as Cd^{2+} or Co^{2+}.

Recently, Lee & Tsien (1982, 1983) have suggested that the blocker-sensitive outward current reflects outward movement of Cs^+ through Ca^{2+} channels. It has also been suggested that Cs^+ can flow outward through Ca^{2+} channels of bovine chromaffin cells (Fenwick, Marty & Neher, 1982). However, this does not seem to be the case under the conditions of our experiments.

First, there is no close correlation between variations in inward Ca^{2+} current at lower potentials and variations in the time-dependent outward current above the apparent 'reversal' potential (Figs. 9, 10*A* and *B*). Secondly, in contrast to the finding by Lee & Tsien (1982), conditioning pre-pulses to inactivate Ca^{2+} channels, which caused a marked reduction in inward current at low potentials, had little effect on the outward current at more positive potentials (Fig. 10*C*). Thirdly, there appear to be two components to the blocker-sensitive current, and the late component reverses at a potential 10–20 mV less positive than the initial component. It is unlikely that this simply reflects inaccurate measurement of the small difference current because at voltages between the reversal potentials for initial and late difference current, the difference current is first inward and then becomes outward. This switching of current direction at a constant voltage indicates involvement of more than one species of conductance. These results force us to conclude that the Cd^{2+}-sensitive outward current seen at large positive potentials is carried by Cs^+ flowing through a voltage-dependent conductance other than Ca^{2+} channels, as previously described in *Helix* and *Limnea* neurones (Kostyuk, Krishtal & Shakhovalov, 1977; Brown, Morimoto, Tsuda & Wilson, 1981; Byerly & Hagiwara, 1982). Because this outward current persists when Ca^{2+} channels are blocked by Cd^{2+} and when currents in

inwardly rectifying K^+ channels are abolished by superfusion with K^+-free solution, we propose that it represents a voltage-dependent leakage current and that, consequently, subtraction of linear leakage currents in order to reveal Ca^{2+} currents is, at best, a hazardous procedure.

The persistence of this leakage current makes it difficult to determine accurately the reversal potential for the Ca^{2+} channel current. The Cd^{2+}-sensitive peak current reverses at $+76 \pm 5$ mV in our experiments ($n = 10$). The presence of the Cd^{2+}-sensitive outward current component discussed above implies that the true reversal potential for Ca^{2+} channel current is expected to be more positive than +76 mV, even if Cs^+ can flow out through the Ca^{2+} channel. This observed reversal potential can therefore be used to obtain a lower estimate of the Ca^{2+}:Cs^+ permeability ratio for the Ca^{2+} channel on the basis of constant field theory (see eqns. (2) and (4) in Meves & Vogel, 1973). If we assume that the intracellular Ca^{2+} activity was lowered to 0·01 μM by 5 mM-EGTA contained in the internal solution, that the extracellular activity coefficient for Ca^{2+} was 0·33 (Shatkay, 1968), that the difference between internal and external surface potentials was 0 mV, that intracellular Cs^+ concentration was the same as that of the pipette solution, and that the intracellular activity coefficient for Cs^+ was 0·75, then the Ca^{2+}:Cs^+ permeability ratio turns out to be $\sim$ 10000.

If K^+ is much more permeable than Cs^+ and carries much larger outward current through the Ca^{2+} channel, replacement of internal K^+ with Cs^+ should have increased the amplitude of the Ca^{2+} channel current. However, this was not the case as shown in Fig. 6, suggesting that the Ca^{2+}:K^+ permeability ratio is also high and that K^+ does not contribute significant charge to the Ca^{2+} channel current.

Sensitivity of Ca^{2+} channel current to external Na^+

In multicellular cardiac preparations, a variety of effects on the Ca^{2+} channel current of reducing external Na^+ have been reported (for review see Coraboeuf, 1980). In mammalian ventricular muscle, for example, Ca^{2+} channel current was, reportedly, unaffected by lowering the Na^+ concentration to 10 % of the normal level, in the cat (New & Trautwein, 1972), while peak Ca^{2+} channel current was decreased by 33 % by reducing the Na^+ concentration from 149·3 to 72·3 mM in bovine heart (Reuter & Scholz, 1977). The sensitivity to Na^+ reduction has been observed also in the sinus node (Noma *et al.* 1977). In frog atrial fibres, Ca^{2+} channel current has been reported to be both sensitive (Lenfant *et al.* 1972), and insensitive (Benninger, Einwächter, Haas & Kern, 1976), to changes in external Na^+.

It has sometimes been supposed that the amplitude of the Ca^{2+} channel current might be reduced on lowering external Na^+ concentration because Na^+ carry charge through the Ca^{2+} channel. However, it is possible that this suppression of Ca^{2+} channel current results from an increase in the intracellular Ca^{2+} concentration via the Na^+–Ca^{2+} exchange mechanism (e.g. Mullins, 1981). This idea is supported by the observation that in non-dialysed single cells, after recovery from a strong contracture induced by Na^+ removal, at which time, presumably, intracellular Ca^{2+} concentration has declined again to a low level, the amplitude of the Ca^{2+} current was the same amplitude as before Na^+ removal (Isenberg, 1982; Hume & Giles, 1983). In the present study we kept the intracellular Ca^{2+} concentration at a low level by adding

5 mM-EGTA to the pipette solution and confirmed that the Ca^{2+} channel current is insensitive to complete removal of external Na^+. On the assumption that removal of external Na^+ does not alter the ionic selectivity of Ca^{2+} channels, this finding would indicate that, under physiological conditions, Na^+ do not contribute as charge carriers to the Ca^{2+} channel current in guinea-pig ventricular cells. Mitchell *et al.* (1983) obtained the same conclusion by replacing external Ca^{2+} with Sr^{2+} to avoid the involvement of the Na^+–Ca^{2+} exchange mechanism in response to reduction of external Na^+.

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